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Protective effect of Mediterranean fish oil extracts on heat-induced denaturation of albumin

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Abstract

Three oily extracts, obtained by acetone extraction from the entrails of different varieties of Mediterranean fishes, such as mackerel (*Scomber scombrus*), sardine (*Sardina pilchardus*) and horse mackerel (*Trachurus mediterraneus*), were characterized to determine their unsaturated fatty acid content. In an in-vitro model, their inhibitory effect was then evaluated against protein aggregation and their protective efficacy against heat-induced albumin denaturation assessed. The fish oil extracts tested in this study presented a significant amount of unsaturated fatty acids; in particular the extract obtained from the entrails of horse mackerel proved to have higher concentrations of DHA (docosahexaenoic acid) and oleic acid compared with the other two oils. The in-vitro study revealed an interesting protective effect of the oil extracts (particularly the horse mackerel extract) against heat-induced denaturation of albumin.

Introduction

During the past 15 years, several studies have investigated the effects of dietary fatty acids on human health (Lombardo & Chicco 2006; MacLean et al 2006). Most of those studies focused on the importance of supplementing diets with fish or vegetable oils containing different, heterogeneously distributed lipid species (Llor et al 2003). Some lipid classes are considered beneficial for human health, such as the polyunsaturated ω-3 fatty acids EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), which are useful in an enormous variety of human diseases (Kremer et al 1990; Carroll 1991), and the monounsaturated ω-9 fatty acids, endowed with an interesting antioxidant activity (Llor et al 2003).

The effect of these lipids on the human immune and inflammatory response has recently been investigated. Different scientific works affirm that either dietary supplementation or topical application of polyunsaturated ω-3 fatty acids could be helpful against chronic inflammatory diseases such as arthritis (Kremer et al 1990). Berbert et al (2005) demonstrated that olive oil (a source of oleic acid, a monounsaturated ω-9 fatty acid) and fish oil (particularly rich in polyunsaturated ω-3 fatty acids) act synergistically when used as dietary constituents or supplements, producing a decrease of inflammatory responses in patients affected by rheumatoid arthritis.

Rheumatic diseases (such as rheumatoid arthritis) belong to the so-called protein condensation diseases and are often characterized by an increased alteration of endogenous proteins due to various agents and conditions. Several non-steroidal anti-inflammatory drugs (NSAIDs) and many endogenous substances, such as inorganic salts, amino acids and different kinds of fatty acids, are capable of protecting globular proteins against denaturation thus beneficially affecting chronic degenerative diseases (Saso et al 1999).

Since different scientific reports point out that polyunsaturated fatty acid (PUFA) supplementation acts positively against rheumatic diseases, it becomes interesting to look into the mechanism employed by these fatty acids to produce these effects and to study their possible role in inhibiting protein aggregation.

In view of this, in this study, we employed three oily extracts obtained by acetone extraction from the entrails of different varieties of Mediterranean fishes, such as mackerel (*Scomber scombrus*), sardine (*Sardina pilchardus*) and horse mackerel (*Trachurus mediter-*

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raneus). The first part of our research was focused on the qualitative and quantitative characterization of the oily extract to obtain information about their fatty acid content and chemical composition. Since substances capable of preventing protein changes could be of potential value in rheumatic conditions, we evaluated, in an in-vitro model, the inhibitory effect of these oil extracts against protein aggregation, thus assessing their protective efficacy against heat-induced albumin denaturation.

Materials and Methods

Materials

Mackerel, sardine and horse mackerel were purchased from a local store. The oil of menhaden fish (*Brevoortia tyrannus*) and linolenic acid, used as reference materials, were purchased from Sigma-Aldrich (Milan, Italy). All the other reagents were of analytical grade.

Fish oil extraction and characterization

The oils were extracted from the fish following a procedure reported previously by us (Puglia et al 2005). The oil samples were obtained from fresh material, quickly stored at −20°C after the extraction process and analysed for their fatty acid content within 48 h. The yield was 6.4% (w/w) for the extract obtained from sardine (**A**), 6.1% (w/w) for the oil extracted from horse mackerel (**B**) and 9.8% (w/w) for the oil extract obtained from mackerel (**C**).

The fatty acid composition of extracts **A**–**C** was determined after methyl esterification according to AOAC methods (Firestone 1995) using the gas chromatographic conditions reported elsewhere (Puglia et al 2005). Identification was effected by comparison with the retention time of methyl ester standards (Supelco PUFA marine oil) and all values represent the mean of three analytical replications (three different portions of the same sample).

Heat-induced denaturation of bovine serum albumin (BSA)

The in-vitro assay of the protective efficacy of the fish extracts against heat-induced albumin denaturation was performed as described by Saso et al (1999). Briefly, each oil was dissolved in 0.066 M phosphate pH 5.3 at 25°C, serially diluted and mixed 1:1 with 0.4% w/v bovine serum albumin (BSA) in the same buffer. Samples of 0.1 mL were pipetted in quadruplicate onto a 96-well microplate (Codisan, Catania, Italy) and the absorbance of the sample was determined at 595 nm. The plate was covered with aluminium foil and incubated at 70 ± 0.1°C for 30 min, and then the absorbance was determined again at the same wavelength. The following formula (Saso et al 1999) was used to calculate the anti-denaturant activity (a_x):

$$a_x = \left[\frac{1 - (A_{x,\,70°C} - A_{0,\,22°C})}{(A_{0,\,70°C} - A_{0,\,22°C})} \right] \times 100 \qquad (1)$$

where $A_{x,\,70°C}$ is the absorbance at 595 nm of the BSA solution heated in the presence of the oil (X); $A_{0,\,22°C}$ and $A_{0,\,70°C}$ are the absorbance of BSA solutions before and after the heating, respectively.

Statistical analysis

The concentrations required for 50% inhibition of heat-induced BSA denaturation (mean stabilizing concentration, SC50) and 95% confidence limits were calculated by using the Litchfield and Wilcoxon test. The results were analysed by the Kruskal–Wallis test; $P < 0.05$ was considered significant. As to the repeatability of the method, the relative standard deviation calculated on four independent replicates of control samples (BSA not added with fish extracts or reference materials) was 2.8%, indicating that the repeatability of the method was good.

Results and Discussion

Table 1 reports the fatty acid composition of oils **A**–**C** (expressed as % areas). The GC analyses showed that all the extracts possessed appreciable amounts of EPA and DHA; in particular DHA concentration in all the extracts was higher than the amount of EPA and the ω-3 fatty acid content was higher than the total ω-6 amount, confirming the information reported in the literature (Passi et al 2002). Furthermore, a large amount of palmitic acid (C16:0) and monounsaturated (MUFA) oleic (C18:1 n-9) acid was present in all the extracts.

Table 2 carries the results regarding the inhibitory effects of the three fish oil extracts on BSA heat-induced denaturation.

Table 1 Fatty acid composition of oil extracts **A**–**C** (% areas)

Fatty acids	Extract A (*Sardina pilchardus*)	Extract B (*Trachurus mediterraneus*)	Extract C (*Scomber scombrus*)
C14:0	6.06 ± 0.28	2.90 ± 0.09	4.91 ± 0.08
C16:0	21.96 ± 0.11	19.09 ± 0.13	22.70 ± 0.14
C16:1	7.21 ± 0.16	6.32 ± 0.09	3.46 ± 0.11
C18:0	0.74 ± 0.01	0.32 ± 0.02	1.61 ± 0.35
C18:1n9	8.92 ± 0.04	22.10 ± 0.06	15.88 ± 0.16
C18:1n7	3.34 ± 1.02	2.78 ± 0.02	3.32 ± 0.01
C18:2n6	1.53 ± 0.13	1.13 ± 0.14	1.74 ± 0.01
C18:4n3	2.46 ± 0.08	0.75 ± 0.01	0.88 ± 0.03
C20:1n9	0.83 ± 0.10	0.48 ± 0.04	1.57 ± 0.01
C20:5n3 (EPA)	10.18 ± 0.66	8.74 ± 0.04	6.79 ± 0.12
C22:1n11	0.55 ± 0.11	nd	0.41 ± 0.17
C22:1n9	0.38 ± 0.08	nd	nd
C22:5n3	1.44 ± 0.16	2.51 ± 0.03	1.64 ± 0.14
C22:6n3 (DHA)	15.91 ± 0.22	24.14 ± 0.08	16.52 ± 0.17
C24:1n9	0.92 ± 0.17	nd	0.33 ± 0.09
Total SFA*	28.72	22.29	29.20
Total MUFA	21.99	31.60	24.97
Total PUFA	31.39	34.67	27.49
PUFA/SFA	1.09	1.50	0.94
PUFA/MUFA	1.42	1.09	1.10

Data are expressed as mean ± s.d. of three analytical replications. *SFA = saturated fatty acids.

Table 2 Effect of fish oil extracts on heat-induced denaturation of bovine serum albumin

Treatment	SC50 ($\mu g\,mL^{-1}$) (95% CL)
Linolenic acid	37.50 (31.80–44.27)
Menhaden fish oil	766.30 (468.92–1252.26)
Extract **A**	252.02 (189.57–335.04)
Extract **B**	49.79 (38.70–64.05)*§
Extract **C**	283.50 (181.98–441.67)

The results are expressed as mean stabilizing concentration (SC50) and 95% confidence limits (CL). *$P < 0.05$ versus extract **A**; §$P < 0.05$ versus extract **C**.

All the extracts proved to have, in different degree, an appreciable protective effect, which was superior to that elicited by the menhaden fish oil used as reference product. Interestingly the extract obtained from *T. mediterraneus* showed the highest activity and its SC50 (mean stabilizing concentration) values were similar to those obtained with linolenic acid. This result can probably be referred to the fatty acid composition of **B** extract (Table 1), which was characterized by a higher DHA and oleic acid content in comparison with the other extracts.

DHA has been reported to affect many biological processes causing the prevention of numerous human diseases (Stillwell & Wassall 2003). The role of DHA in these processes, often unrelated to each other, is probably due to its chemical structure and particularly to its high degree of conformational flexibility conferred by the multiple double bonds. The DHA beneficial action is linked to the interaction with membrane lipids, particularly cholesterol, and to the modulation of the activity of some protein classes (Stillwell & Wassall 2003). Some researchers have hypothesized that DHA might possibly alter the protein conformation (Pascale et al 1993) and, in some cases, increase the efficacy and the stability of specific protein structures (Siddiqui et al 2003).

However, apart from the DHA, the protective effect of extract **B** against protein denaturation could be justified by other hypotheses. The appreciable amount of oleic acid, in fact, could participate in the net protective effect of extract **B**. Oleic acid is an ω-9 monounsaturated fatty acid (C18:1 ω-9) that is rapidly converted, after supplementation, to eicosatrienoic acid (ETA; C20:3 ω-9), a potent inhibitor of leukotriene B_4 synthesis. Berbert et al (2005) demonstrated that ETA may exert its anti-inflammatory action through a mechanism analogous to that of fish oil, thus confirming the results as to the antioxidant and protective activity of oleic acid pointed out by different in-vitro and in-vivo studies (Hashim et al 2005). Thus, one could speculate that the protective effect of extract **B** is markedly higher than that induced by the other extracts due to its peculiar qualitative and quantitative chemical profile, particularly rich in two fatty acids, DHA and oleic acids, which might also act synergistically. Furthermore, our results outlined that in all the oil extracts the MUFA/PUFA ratio had no significant effect, thus showing that the protective effect obtained in the in-vitro model was not related to this data.

In conclusion, the fish oil extracts tested in this study presented a significant amount of unsaturated fatty acids, extract **B** DHA and oleic acid concentrations being higher than those of the other two extracts. An interesting protective effect of the oil extracts against heat-induced denaturation of BSA was also demonstrated; extract **B** showed the best inhibitory activity, very likely due to its abundant content of DHA and oleic acid.

The anti-denaturant properties of these extracts may be useful either to develop new therapeutic tools for selected diseases in which protein denaturation plays a pathological role or to increase the anti-inflammatory activity of NSAIDs, commonly administered to patients with rheumatic disorders.

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